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Bone Marrow Stromal Cell-Mediated Gene Therapy for Hemophilia A: *In Vitro* Expression of Human Factor VIII with High Biological Activity Requires the Inclusion of the Proteolytic Site at Amino Acid 1648

GISELA G. CHIANG,[1] HILLARD L. RUBIN,[1] VAN CHERINGTON,[1] TI WANG,[1] JOYCE SOBOLEWSKI,[1] CLAIRE A. McGRATH,[1] ANITA GAFFNEY,[1] SHAHIN EMAMI,[2] NAVA SARVER,[3] PETER H. LEVINE,[4] JOEL S. GREENBERGER,[1] and DAVID R. HURWITZ[1]

ABSTRACT

To evaluate the potential of the *ex vivo* bone marrow stromal cell (BMSC) system as a gene therapy for hemophilia A, we studied the *in vitro* expression of human factor VIII (hFVIII) in canine BMSCs following transfection with plasmid vectors and transduction with retroviral vectors. Vectors were composed of B domain-deleted forms of hFVIII that either retain or delete the proteolytic site at amino acid 1648. On transfection of BMSCs, vectors supported expression and secretion of similar levels of up to 386 mU/10^6 cells/24 hr, even though only 3–9% of the cells expressed hFVIII while 42–48% of transfected cells harbored plasmid vector. Much higher percentages ($\sim$70%) of cells expressing hFVIII were achieved when BMSCs were transduced by retroviral vectors, resulting in expression and secretion as high as 1000–4000 mU/10^6 cells/24 hr. Western analysis demonstrated that the B domain-deleted forms possessing the proteolytic site were secreted predominantly as heavy and light chain heterodimers that resemble native forms found in plasma. In contrast, the hFVIII lacking the proteolytic site was expressed mostly as unprocessed, single heavy–light chains. Both hFVIII forms were correctly cleaved and activated by thrombin. The proteolyzed hFVIII form possessed $\geq$93% normal biological activity while the unproteolyzed form possessed consistently less than 55% normal biological activity and was therefore considered less suitable for therapeutic application. These results demonstrate that the BMSC system has potential utility in gene therapy for hemophilia A and stress the importance of selecting the appropriate hFVIII structure for prospective clinical use.

OVERVIEW SUMMARY

Toward the development of the *ex vivo* bone marrow stromal cell system as an efficacious gene therapy for hemophilia A, we have demonstrated high-level expression of two different B domain-deletion forms of hFVIII by plasmid vector-transfected, and retroviral vector-transduced, stromal cells in culture. The B domain-deleted form of hFVIII, which maintains the native proteolytic site at amino acid 1648, exhibits a high percentage of normal biological activity, and is expressed as heavy and light chain heterodimers (similar to native hFVIII). In contrast, the B domain-deleted form, which lacks the proteolytic site, exhibits a limited percentage of normal biological activity and is secreted predominantly as nonproteolyzed, contiguous heavy–light chain monomers. These results demonstrate the potential importance of the stromal cell system in gene therapy for hemophilia and suggest that the former B domain-deleted form of factor VIII may be more appropriate for gene therapy applications than the latter.

[1]ALG Company, Marlborough, MA 01752.
[2]Present address: INSERM-U-482, Centre de Recherches Paris Saint-Antoine, 75571 Paris cedex 12, France.
[3]Division of AIDS, National Institutes of Allergy and Infectious Diseases, Bethesda, MD 20892.
[4]Memorial Health Care, Worcester, MA 01605.

INTRODUCTION

HEMOPHILIA A is a genetic disease that accounts for 85% of all cases of hemophilia and afflicts 10 to 15 males per 100,000 people worldwide (Rosendaal *et al.*, 1991; Brettler and Levine, 1994). The disorder is caused by an X-linked deficiency in coagulation factor VIII (FVIII), an essential cofactor required for factor IX (FIX)-mediated activation of factor X in the intrinsic pathway of coagulation (reviewed in Kaufman, 1992). Present treatment, the frequency of which is dependent on the severity and frequency of hemorrhaging episodes, is limited to infusions of human FVIII (hFVIII) either purified from plasma or derived as a recombinant product. While replacement of plasma-derived hFVIII with recombinant hFVIII circumvents exposure to potential infectious contamination, their clinical use regimens are similar. Factor VIII treatment is sufficient to halt bleeding episodes but it is noncurative. In addition, multiple prophylactic hFVIII infusions may not be feasible owing to high cost, limited plasma resources, and possible increase in side effects (Rosendaal *et al.*, 1991; Brettler and Levine, 1994; Aledort and Bohn, 1996). Thus, a somatic gene therapy providing sustained delivery of therapeutic levels of hFVIII toward prophylactic treatment of hemophilia A would be highly beneficial (Fallaux *et al.*, 1995; Thompson, 1995; Connelly and Kaleko, 1997; Walter and High, 1997).

The biosynthesis, structure, and function of FVIII have been studied in great detail (Kaufman, 1992). The primary translation product of hFVIII is a large (300-kDa), single heavy–light chain monomer spanning approximately 2351 amino acids. It is composed of a multiple-domain structure ordered as A1-A2-B-A3-C1-C2 (Vehar *et al.*, 1984). The B domain is defined at its N terminus by a thrombin-sensitive site at amino acid (aa) 740 and by a proteolytic processing site at aa 1648 at its C terminus. Processing of FVIII begins in the Golgi apparatus, in which proteolysis at aa 1313 (within the B domain) and aa 1648 releases the 80-kDa light chain from the heavy chain (Kaufman *et al.*, 1988; Pittman and Kaufman, 1988; Kaufman, 1992). Factor VIII purified from plasma is present as cleaved heterodimers containing the carboxyl-terminal light chain, Ca^{2+} abridged to the amino-terminal, heterogeneously sized (90- to 200-kDa) heavy chain, which arises from variable proteolysis of the B domain (Andersson *et al.*, 1986; Eaton *et al.*, 1986a). Factor VIII undergoes activation in the plasma by thrombin cleavage at aa 372, 740, and 1689, and complete removal of the B domain. Cleavage at aa 372 and 1689 by thrombin is essential for activation (Pittman and Kaufman, 1988). Cleavage at aa 740 releases a 90-kDa heavy chain. Activation by thrombin cleavage of the heavy chain at positions 372 and 740 generates 50- and 43-kDa fragments, and cleavage of the light chain at position 1689 generates a 73-kDa fragment.

Use of an *ex vivo* gene therapy strategy represents one approach toward a gene therapy for hemophilia (Boulikas, 1996; Connelly and Kaleko, 1997). Such an approach is defined as the *in vitro* genetic modification of cells, which when transplanted *in vivo* allow continuous delivery of therapeutic product. Bone marrow stromal cells (BMSCs) offer one potential cell type for the introduction and delivery of transgenes and their products (Greenberger, 1996; Prockop, 1997). Genetically modified BMSCs have been shown to express major histocompatibility class II protein (Huss *et al.*, 1995), extracellular matrix human collagen type I (Pereira *et al.*, 1995), interleukin 3 (Nolta *et al.*, 1994), and human FIX (hFIX) (Guinn *et al.*, 1996) in mice on transplantation. Our initial investigations toward developing a BMSC-based *ex vivo* somatic therapy defined conditions for establishing and expanding BMSCs to large numbers ($>10^9$) (Emami *et al.*, 1997). These cells were characterized as a homogeneous population of bone marrow myofibroblasts lacking in endothelial cells, macrophages, and hematopoietic progenitor cells. In developing a BMSC-based therapy for hemophilia B, we demonstrated that canine BMSCs, genetically modified *ex vivo* either by plasmid vector transfection or retroviral vector transduction, delivered human blood coagulation factor IX to the systemic circulation of dogs (Hurwitz *et al.*, 1997; Chiang *et al.*, 1998). More recently, we demonstrated transient *in vivo* therapeutic plasma levels of human factor IX (Cherington *et al.*, 1998). The transient nature of expression in this latter study could be attributed to the demonstrated generation of canine anti-hFIX antibodies (Cherington *et al.*, 1998). Vector-modified cells were detectable for as long as 6 months following autologous infusion in dogs (Cherington *et al.*, 1998; Chiang *et al.*, 1998).

We now extend our studies to the *in vitro* expression of hFVIII in BMSCs to evaluate the utility of a BMSC-mediated gene therapy for hemophilia A. It has previously been demonstrated that FVIII activity was retained, and in some cases increased, in recombinant FVIII forms that lacked most or all of the B domain (Eaton *et al.*, 1986b; Toole *et al.*, 1986; Sarver *et al.*, 1987; Meulien *et al.*, 1988; Hoeben *et al.*, 1990; Bihoreau *et al.*, 1991; Dwarki *et al.*, 1995; Lind *et al.*, 1995). Whether resultant expressed forms were predominantly heterodimers of heavy and light chain, or single heavy–light contiguous peptide chains, was largely dependent on the presence or absence of the proteolytic site at aa 1648. Reports on *in vivo* studies in dogs, and human clinical trials, show that the pharmacokinetics, efficacy, and safety of B domain-deleted heterodimeric recombinant FVIII forms were equivalent to those of plasma-derived forms (Pittman *et al.*, 1993; Berntorp, 1997). High levels of a largely single-chain hFVIII configuration have been produced from retroviral vector-transduced primary human fibroblasts (Dwarki *et al.*, 1995) and human BMSCs (Chuah *et al.*, 1998). Vaccinia virus expression of another principally single-chain hFVIII has been suggested to be more stable or active than that of native hFVIII (Meulien *et al.*, 1988).

To compare directly the expression capacities and properties of heterodimeric versus single-chain secreted forms of hFVIII in the BMSC model, we constructed expression plasmids for two B domain-deleted forms of hFVIII that encode for either the presence or absence of the proteolytic site at aa 1648. These plasmid vectors were used to generate retroviral vectors. We demonstrate that BMSCs secrete biologically active hFVIII following transfection and further demonstrate high expression rates of hFVIII following efficient retroviral vector transduction. Our results demonstrate the potential use of BMSCs for gene therapy of hemophilia A. The two different deleted hFVIII forms displayed significantly different percent biological activities, which were greater in hFVIII retaining the proteolytic site at aa 1648. These results imply a therapeutic advantage of this form for clinical application.

MATERIALS AND METHODS

In vitro *establishment and culturing of canine bone marrow stromal cells*

Canine BMSCs were established, cultured, and cryopreserved as described previously (Emami *et al.*, 1997; Hurwitz *et al.*, 1997; Cherington *et al.*, 1998). Complete BMSC medium is composed of Dulbecco's modified Eagle's medium (DMEM) containing amphotericin B (Fungizone; 0.25 μg/ml), gentamicin (25 μg/ml), penicillin (100 units/ml), streptomycin sulfate (100 μg/ml), 16% heat-inactivated fetal bovine serum (FBS), human recombinant acidic fibroblast growth factor (aFGF; 1 ng/ml), and sodium heparin (5 units/ml) (Elkins-Sinn, Cherry Hill, NJ).

Construction of B domain-deleted hFVIII (pMFG-hFVIIIΔB) plasmid vectors

Retroviral plasmid vector MFG-cFMS was kindly provided by P. Robbins (University of Pittsburgh School of Medicine). The MFG vector backbone (Dranoff *et al.*, 1993) was separated from the c-*fms* sequences by restriction enzyme digestion using *Nco*I and *Bam*HI. The three versions of B domain-deleted hFVIII cDNA encoding $hFVIII_G\Delta B_{814}$, $hFVIII_E\Delta B_{814}$, and $hFVIII_E\Delta B_{906}$, protein molecules were derived from plasmid p809 (D.R. Hurwitz and N. Sarver, unpublished), a derivative of previously described hFVIII expression plasmids (Sarver *et al.*, 1987), and cloned into MFG retroviral vector. Plasmid p809 contains the *Xho*I subcloned fragment of hFVIII cDNA encoding a partially deleted B domain (deleted residues 747–1560, numbered according to Toole *et al.*, 1984), which includes an additional 26 and 215 base pairs (bp) of 5′ and 3′ untranslated (UTR) sequences, respectively.

Construction of pMFG-hFVIII$_G\Delta B_{814}$

Protein $hFVIII_G\Delta B_{814}$ contains a B domain deletion of 814 aa. It contains a modified 5′ translation initiation sequence to match more closely the Kozak translational consensus sequence GCC(ATG)G (Kozak, 1989) and therefore encodes a glycine (G) at the second amino acid position (numbered according to Toole *et al.*, 1984) of the secretory signal sequence. The 4.8-kb fragment encoding the B domain-deleted hFVIII sequence was excised from p809 by *Xho*I restriction enzyme digestion, subcloned into pLITMUS 28 (New England BioLabs, Beverly, MA), and designated pF8-4.8. The upstream −20 to 1839 nucleotides (nt) of hFVIII was removed from pF8-4.8 by digesting with *Kpn*I followed by religation to create pF8-3′KX. To remove the 3′ UTR, a polymerase chain reaction (PCR) fragment was amplified from p809 using 5′ primer (5′-GTGGTGAACTCTCTAGACCCAC) and 3′ primer (5′-GGTAGATCTTCAGTAGAGGTCCTGTG), which incorporated an *Xba*I site at the 5′ end and added a *Bgl*II site immediately following the translation stop codon. This PCR insert was subsequently used to replace the 3′ *Xba*I–*Bgl*II fragment of pF8-3′KX and generate pF8-3′KB. Plasmid vector pMFG-$hFVIII_G\Delta B_{814}$ was assembled by a three-way ligation using the *Nco*I–*Kpn*I-flanked 1840-bp fragment from pF8-4.8 containing upstream FVIII cDNA sequences, the 2794-bp *Kpn*I–*Bgl*II fragment derived from pF8-3′KB, and the *Nco*I–*Bam*HI-linearized MFG retroviral vector backbone. Plasmid vector pMFG-$hFVIII_G\Delta B_{814}$ retains sequences encoding the first six amino acid residues of the B domain following the thrombin cleavage site at residue 740, and 87 amino acid residues prior to the proteolytic cleavage site at residue 1648. All junction sites and PCR-generated inserts used for cloning were confirmed by sequence analysis.

Construction of pMFG-hFVIII$_E\Delta B_{814}$

Plasmid pMFG-$hFVIII_G\Delta B_{814}$ was altered by replacement of nucleotide G with A at position +5 to optimize further the Kozak consensus sequence GCC(ATG)GA (Grünert and Jackson, 1994). The nucleotide switch results in the conversion of the second amino acid of the signal peptide from glycine (G) to glutamic acid (E) and was generated by PCR amplification from template pF8-4.8 using 5′ primer (5′-GGACCATGGAAATAGAGCTC) and 3′ primer (5′-GGTACTAGTAGGGCTCCAATG), which incorporated a 5′ *Nco*I site and 3′ *Spe*I site. The *Nco*I–*Spe*I PCR product was coligated with a 1286-bp *Spe*I–*Bam*HI hFVIII fragment from pF8-4.8 into the *Nco*I–*Bam*HI sites of LITMUS 28 and designated pF8-5′Glu. Plasmid vector pMFG-$hFVIII_E\Delta B_{814}$ was assembled by a three-way ligation using the *Nco*I–*Kpn*I-flanked 1840-bp fragment with pF8-5′Glu containing upstream FVIII cDNA sequences, the 2794-bp *Kpn*I–*Bgl*II fragment derived from pF8-3′KB containing downstream hFVIII sequences, and the *Nco*I–*Bam*HI-linearized MFG retroviral vector backbone. All junction sites used for cloning and PCR-generated inserts were confirmed by sequence analysis.

Construction of pMFG-hFVIII$_E\Delta B_{906}$

The $hFVIII_E\Delta B_{906}$ protein contains a B domain deletion of 906 aa. It is also composed of the optimized Kozak consensus sequence and therefore encodes a glutamic acid (E) in the second amino acid position of the signal peptide. Vector pMFG-$hFVIII_E\Delta B_{906}$ was derived from pMFG-$hFVIII_G\Delta B_{814}$ by deletion of DNA sequences encoding almost the entire B domain (residues 743–1648), resulting in removal of an additional 92 amino acids. A 601-bp PCR product was generated using 5′ primer p10 (5′-TCCAAGCTTCGAAATAACTCGTACTACT) and 3′ primer p9 (5′-TTTTTCCAGGTCAACATC). Primer p10 encodes a fusion of the first two amino acids of the B domain (residues 740–741) with amino acids immediately following the B domain junction (residues 1649–1655). The resulting PCR product was isolated as a *Hind*III–*Nde*I fragment. The DNA encoding the 92 deleted amino acids of the B domain was excised from vector pF8-3′KB by *Hind*III/*Nde*I restriction enzyme digestion and replaced with the *Hind*III–*Nde*I PCR product to generate pF8-3′KBΔB.

Plasmid pF8-3′KBΔB was subsequently digested with *Kpn*I/*Bgl*II and the 2518-bp fragment isolated. Vector pMFG-$hFVIII_G\Delta B_{906}$ was assembled by a three-way ligation using the 2518-bp *Kpn*I–*Bgl*I fragment isolated from pF8-3′KBΔB, the *Nco*I–*Kpn*I 1840-bp fragment from pF8-5′Glu (containing the Kozak like sequence), and the *Nco*I–*Bam*HI-linearized MFG retroviral vector backbone. Vector-encoded plasmid MFG-$hFVIII_E\Delta B_{906}$ retains the first two amino acids of the B domain following the thrombin cleavage site at residue 740 and lacks the proteolytic site at residue 1648. All junction sites used for

cloning and PCR-generated inserts were confirmed by sequence analysis.

In vitro *transfection of canine BMSCs with human FVIII retroviral plasmid vectors*

Canine BMSCs seeded at $\sim 3 \times 10^5$ cells in 25-cm^2 flasks were transfected with retroviral vector plasmids by the cationic lipid–DNA complex method using LipofectAMINE reagent and Opti-MEM reduced serum medium (Life Technologies, Gaithersburg, MD) (20 μg of each plasmid made up in a total volume of 5.66 ml of Opti-MEM containing 0.33 ml of LipofectAMINE reagent and incubated with cells for 5–6 hr). In some cases cells were transfected two or three times, with 3 to 4 days in between transfection rounds.

Packaging cell lines, generation and screening of retroviral vector-producing clones

Ecotropic packaging cell line GP+E-86 (Markowitz *et al.*, 1988a) and amphotropic packaging cell line GP+envAM12 (Markowitz *et al.*, 1988b) were generously provided by Genetix (New York, NY) and cultured as described previously (Cherington *et al.*, 1998).

MFG-hFVIII$_G\Delta B_{814}$ and MFG-hFVIII$_E\Delta B_{906}$ retroviral vector GP + envAM12 producer clones used in this study were generated as follows. GP+E-86 ecotropic cells were cotransfected with plasmids pMFG-hFVIII$_G\Delta B_{814}$ and pSV2neo or with plasmids pMFG-hFVIII$_E\Delta B_{906}$ and pSV2neo by the cationic lipid–DNA complex method using LipofectAMINE reagent and Opti-MEM I reduced serum medium (Life Technologies). Polyclonal populations of transfected GP+E-86 ecotropic cells were selected in G418 (Geneticin, 1 mg/ml; Life Technologies). MFG-hFVIII$_G\Delta B_{814}$ and MFG-hFVIII$_E\Delta B_{906}$ GP+envAM12 producer cells were generated by transducing GP+envAM12 cells multiple times with viral supernatant from ecotropic GP+E-86 producer cells. Amphotropic producer cell clones were subsequently isolated by limited dilution in 96-well plates and by assaying hFVIII production levels from clonal lines by enzyme-linked immunosorbent assay (ELISA) and one-stage procoagulant assay. Amphotropic producer clones secreting the highest concentration of functionally active hFVIII were further expanded. To screen for the production of functionally active retroviral vector, viral supernatants from producer clones were used to transduce canine BMSCs. Optimal hFVIII producer clones were selected on the basis of their capacity to transduce stromal cells resulting in the production of significant levels of hFVIII. Clones designated H20 and D22 derived from pMFG-hFVIII$_G\Delta B_{814}$ and pMFG-hFVIII$_E\Delta B_{906}$, respectively, were chosen from this first round of screening. Correct hFVIII secretion and processing by BMSCs transduced by these clones was confirmed by Western analysis. Final clones reported in this study (C45 and C25 of MFG-hFVIII$_G\Delta B_{814}$, 10B6 and 10D10 of MFG-hFVIII$_E\Delta B_{906}$) were generated by using amphotropic viral supernatants from H20 and D22 to transduce ecotropic producer cell cultures and vice versa in a ping–pong method, followed by isolation after a further two rounds of screening.

In vitro transduction of canine BMSCs with human FVIII retroviral vectors from producer clones

Viral supernatants were prepared from producer clones expanded in 150-cm^2 flasks to 70–80% confluence at 37°C, 5% CO_2. Cells were overlaid with 15 ml of fresh medium composed of DMEM supplemented with 10% heat-inactivated newborn calf serum. After 24 hr, viral supernatant was collected, passed through 0.22-μm pore size filters, and either used fresh or flash frozen in liquid nitrogen and stored at −80°C for later use. Supernatants supplemented with Polybrene (8 μg/ml) were used to transduce 2.5×10^5 BMSCs seeded the day before in 25-cm^2 flasks. After at least 6 hr, virus was removed and complete BMSC medium added. The transduction procedure was repeated two to four times on consecutive days. When transduced cells had expanded to $\sim$80% confluence, medium was changed to determine expressed hFVIII levels after 24 hr.

In vitro *hFVIII:c assays and determination of percent normal human plasma biological activity*

Conditioned media from transfected and transduced BMSCs were harvested to quantify hFVIII procoagulant activity and antigen levels. Since aliquots of the same samples were subjected to both ELISA and procoagulant activity assays, the medium used during conditioning for these samples was adjusted to accommodate both assays. The serum in the medium was lowered to 10% (transfected cells) or 5% (transduced cells) in order to reduce the background in the ELISAs. BMSC cell growth, and final procoagulant and ELISA hFVIII antigen levels over 24 hr, were not different between media conditioned in the presence of 10 or 5% FBS (data not shown). In addition, the heparin supplement was omitted in the conditioning medium or it would have inhibited procoagulant activity assays. Medium was conditioned for 24 or 48 hr (transfected cells) or for 24 hr (transduced cells).

Human FVIII biological activity in the conditioned medium was determined by the one-stage procoagulant assay performed on the Automated Coagulation Laboratory (ACL)-3000 plus (Instrumentation Laboratories, Lexington, MA), which measures the time to develop a fibrin clot after addition of specific reagents. The sensitivity of the assays is approximately 30 mU/ml (3% activity). Normal pooled human plasma (George King Biomedical, Overland Park, KS) was used as the activity standard and was assumed to contain 200 ng of hFVIII per milliliter. A-FACT (abnormal at 7%) and B-FACT (borderline at 42%) (George King Biomedical) plasma controls were included in each assay run to validate coagulation tests. By definition, 1 unit of FVIII activity is equivalent to the activity of hFVIII:c present in 1 ml of normal pooled human plasma and is defined as 100% clotting activity. Control tissue culture media conditioned by mock-transfected or mock-transduced BMSCs were used for the background control and subtracted from samples derived from hFVIII-transfected or -transduced cells. Background control activities did not exceed 30 mU/ml ($<$3.0%).

A sandwich hFVIII ELISA was developed to quantify BMSC-secreted hFVIII antigen levels in tissue culture. Immunoplate Maxisorp (Nunc, Naperville, IL) multiwell trays were coated with monoclonal anti-human FVIII:c from Boehringer Mannheim (Indianapolis, IN) and ESH 5 monoclonal anti-human FVIII:c from American Diagnostica (Green-

wich, CT), specific for the human light chain and the A1 heavy chain domain, respectively. Secondary antibody horseradish peroxidase (HRP)-conjugated anti-human FVIII:c ESH 8 antibody specific for the hFVIII C2 domain (American Diagnostica) was used to detect bound FVIII antigen. Conditioned tissue culture medium from mock-transduced cells was used as a background as well as for the milieu in which medium samples and hFVIII assay standards were diluted. A four-parameter standard curve ranging from 0.781–1.562 to 200 ng/ml was generated using normal pooled plasma (George King Biomedical), assumed to contain 200 ng of hFVIII per milliliter. The sensitivity of the assay for conditioned media containing 10% FBS and 5% FBS was 1.562 and 0.781 ng/ml, respectively.

Percent normal human plasma biological activity of hFVIII secreted by BMSCs was determined following multiple rounds of plasmid transfection in replicate flasks for each vector. Three days following the final transfection, media conditioned for 48 hr (during which accumulation of FVIII activity in conditioned media is linear; data not shown) were collected from cells growing to 80 to 90% density. This was typically a total of 13 to 14 days following seeding. Similarly, medium conditioned for 24 hr was sampled from retrovirus vector-transduced BMSCs, typically 6 days following transduction and a total of 9 days following seeding. The percent normal biological activity of the hFVIII in a conditioned medium sample was calculated by comparing the hFVIII clotting activity with the ELISA-determined hFVIII protein level. Normal pooled human plasma was used as the standard and it was assumed that a normal hFVIII plasma level of 200 ng/ml represents 100% (1 U) normal biological activity.

Activation of human FVIII by thrombin

Human FVIII-containing samples adjusted to approximately equal procoagulant activity were incubated with human thrombin (specific activity, 3000 NIH units/mg: 300 mU/ml, Sigma, St. Louis, MO) at 37°C. At the indicated time intervals, a 10-μl aliquot was diluted 1:20 in test factor diluent (Instrumentation Laboratories) and immediately assayed in the one-stage clotting assay. Normal pooled human plasma (George King Biomedical) was used to generate standard curves whereby procoagulant activity expressed in milliunits per milliliter was plotted as a function of time.

Immunoprecipitation and Western blot analysis of hFVIII

Medium conditioned by transfected or transduced BMSCs expressing hFVIII was clarified by centrifugation and diluted in an equal volume of immunoprecipitation buffer (300 m*M* Tris-HCl [pH 7.4], 0.2% sodium dodecylsulfate [SDS], 2% Triton X-100, 2% sodium deoxycholate) supplemented with leupeptin (1 μg/ml), pepstatin A (1 μg/ml), and 1 m*M* phenylmethylsulfonyl fluoride (PMSF) (Sigma). Factor VIII was immunoprecipitated with monoclonal antibody (MAb) ESH 8 (5 μg/ml), which was coupled to protein G–Sepharose using the ImmunoPure Protein G IgG orientation kit (Pierce, Rockford, IL). Sepharose pellets were washed serially with phosphate-buffered saline (PBS) containing 1, 0.1, and 0.05% Triton X-100, suspended in Tris-buffered saline (TBS), and incubated for 2 min at 37°C in the presence or absence of human thrombin (10 U/ml). Immunoprecipitates were eluted in SDS sample buffer and resolved on a 10% reducing SDS-polyacrylamide gel. Gels were blotted onto nitrocellulose (Schleicher & Schuell, Keene, NH) by electrotransfer. Membranes were blocked in 20 m*M* Tris-HCl, 137 m*M* NaCl, 1% Tween 20 (pH 7), and 5% nonfat milk, then incubated with either mono-clonal antibody maHF8c-ab (Research Diagnostics, Flanders, NJ) specific for the light chain of hFVIII, or an affinity-purified sheep polyclonal anti-hFVIII antibody (Cedarlane Laboratories, Hornby, Ontario, Canada). Washed immunoblots were incubated with either an anti-mouse (0.1 μg/ml; Sigma) or an anti-sheep HRP-conjugated antibody (0.08 μg/ml; Pierce) after which protein bands were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL) according to the manufacturer instructions and visualized by exposure to film (X-Omat AR; Eastman Kodak, Rochester, NY).

Human FVIII vector fluorescence in situ *hybridization analysis and immunofluorescence*

Cultured BMSCs in 25-cm^2 flasks were transfected with vector plasmids in duplicate and procoagulant activity determined as described above. Cultures in one set of flasks were then examined directly by immunofluorescence staining. For the remaining duplicate set of flasks, cells were trypsinized, counted, and immediately centrifuged at 500 rpm for 2 min onto 25 $\times$ 75 mm glass slides, at 5×10^4 cells/slide, using a Cytospin 3 (Shandon, Pittsburgh, PA) in preparation for either fluorescence *in situ* hybridization (FISH) or immunofluorescence analysis.

For examination by immunofluorescence staining, slides were washed in PBS (Life Technologies) and fixed with 4% electron microscopy (EM)-grade paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in PBS for 10 min followed by permeabilization with 0.5% Triton X-100 in PBS for 15 min. Cells were blocked with a 1:200 dilution of normal goat serum (Vector Laboratories, Burlingame, CA) in PBS, then incubated with light chain-specific hFVIII mouse MAb (ESH 8, 1 μg/ml; American Diagnostica) for 60 min. Cells were washed again, followed by incubation with rhodamine red-X goat anti-mouse IgG (10 μg/ml; Molecular Probes, Eugene, OR). For detection of nuclei, cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml; Molecular Probes) for 5 min. After a final wash in PBS sections were air dried, mounted in Fluoromount-G (Southern Biotechnology Associated, Birmingham, AL), and visualized using a Leica Microstar IV phase-contrast fluorescence microscope (Leica AG, Heerbrugg, Switzerland) equipped with a DAPI/FITC/Texas Red tri-color filter (ChromaTechnology, Brattleboro, VT). Negative controls included vector-transfected cells processed through the same protocol with a nonspecific murine $IgG_{2a}(\kappa)$ control antibody (PharMingen, San Diego, CA), as well as the processing of mock-transfected cells using the specific ESH 8 antibody.

For examination by FISH, slides were washed in PBS (Life Technologies) and fixed with 4% EM-grade formaldehyde (Electron Microscopy Sciences) in PBS for 15 min at room temperature. Samples were air dried for 5 min, then incubated with proteinase K (2 μg/ml; GIBCO-BRL, Gaithersburg, MD) for 10 min followed by 0.3% Triton X-100 (Sigma) for 10 min. Slides were postfixed in 2% paraformaldehyde for 2 min fol-

lowed by dehydration in a series of ethanol treatments (50, 70, 90, and 100% each for 2 min at room temperature), denatured with 70% (v/v) formamide–2× standard saline citrate (SSC, pH 7.0) at 75°C for 5 min. Samples were dehydrated again with a series of ethanol treatments (50, 70, 90, and 100%; each for 2 min) at −20°C.

The DNA probe was prepared as follows. Plasmid pMFG-hFVIII$_G\Delta B_{814}$ (1 μg per reaction) was labeled with digoxigenin–11-dUTP using DIG-Nick translation mix (Boehringer Mannheim) according to the manufacturer instructions. Just before use, the probe was denatured by heating at 95°C for 5 min and diluted to 1–5 ng/ml in hybridization buffer (50% deionized formamide, 2× SSC, 10% dextran sulfate, 1× Denhardt's solution, salmon sperm [100 ng/ml], and 50 m*M* sodium phosphate). A volume of 10–20 μl of probe mixture was added to each sample, covered with 22 × 22 mm glass coverslips, and incubated in a humidified chamber at 37°C for 20 hr. Slides were washed twice with 2× SSC for 10 min followed by 0.2× SSC twice for 5 min at room temperature. Samples were briefly soaked in PBS then incubated for 30 min a 37°C with 0.5% blocking reagent (Boehringer Mannheim) in a humidified chamber. The digoxigenin-labeled probes were detected by incubating slides with anti-digoxigenin–fluorescein (Boehringer Mannheim) for 30 min at 37°C followed by two 2-min washes in PBS at room temperature. Counterstaining of nuclei was performed using propidium iodide (Molecular Probes). After a final wash in PBS, slides were air dried, mounted in Fluoromount-G (Southern Biotechnology Associated), and visualized using a Leica Microstar IV phase-contrast fluorescence microscope (Leica AG) in conjunction with a DAPI/FITC/Texas Red tri-color filter (ChromaTechnology). Plasmid-transfected cells processed through the same protocol except in the absence of hybridization probe as well as mock-transfected cells hybridized to probe provided the negative controls.

The percentage of BMSCs harboring vector DNA sequences was determined from the number of cells stained positive for DNA hybridization signals compared with the total number of cells (propidium iodide-stained nuclei) counted. The percentage of BMSCs expressing FVIII was similarly determined from the number of cells stained positive with anti-hFVIII antibody compared with the total number of cells (DAPI-stained nuclei) counted. Average totals of 300 and 1300 cells were counted for FISH and immunofluorescent analysis, respectively, per vector plasmid analyzed.

Statistical analysis

Statistical significance of assay results was determined using Student's *t* test. Groups of unpaired values were defined as not different when $p > 0.05$.

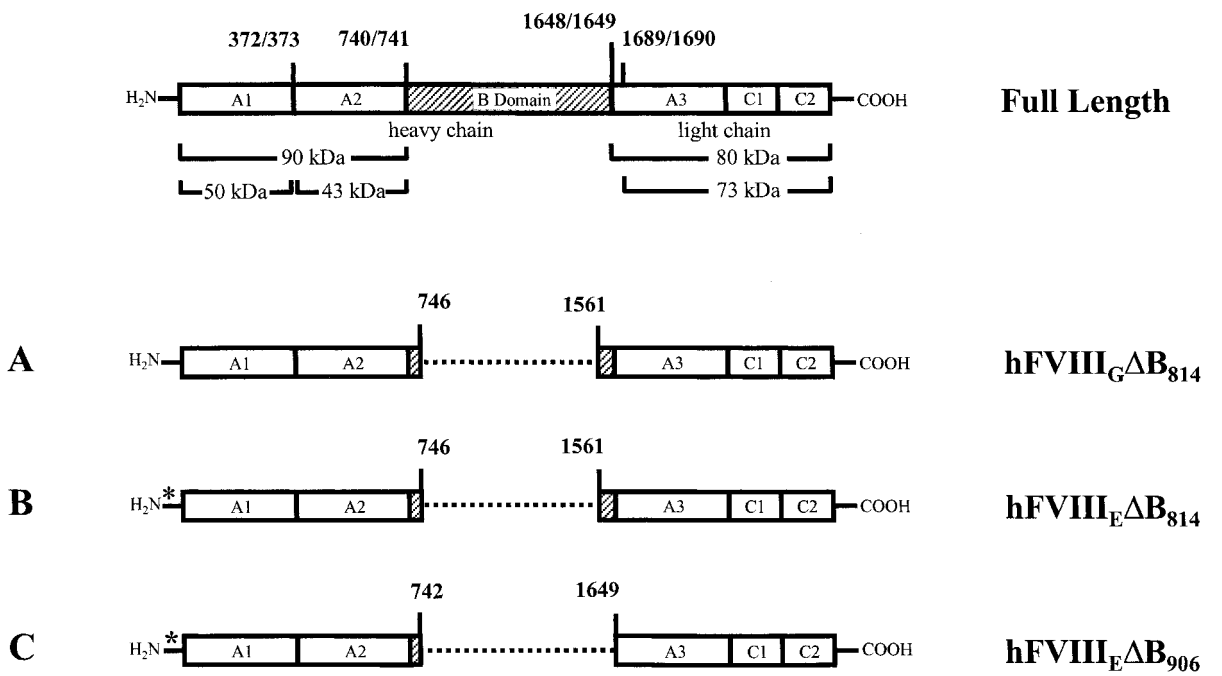

FIG. 1. Schematic representation of B domain-deleted hFVIII protein structures encoded by vectors. *Top:* Full-length hFVIII. Thrombin cleavage sites (aa 372, 740, and 1689) and the proteolytic site at aa 1648 are indicated (Toole *et al.*, 1984). Cleavage at aa 372 and 1689 is necessary for hFVIII thrombin activation. Approximate sizes of domains are shown. Domains A1-A2-B and A3-C1-C2 comprise the heavy and the light chain, respectively. The 50-, 43-, and 73-kDa fragments represent products generated after thrombin cleavage. Protein molecules hFVIII$_G\Delta B_{814}$ (**A**) and hFVIII$_E\Delta B_{814}$ (**B**) lack amino acids 747 to 1560 (a deletion of 814 aa) of the B domain. Protein molecules of hFVIII$_E\Delta B_{906}$ (**C**) lack amino acids 743 to 1648 (a deletion of 906 aa), including the proteolytic site at 1648. Asterisks (*) indicate a vector modification to better match an optimized Kozak translation consensus sequence by replacement of G with A at position +5 in hFVIII$_E\Delta B_{814}$ and hFVIII$_E\Delta B_{906}$, resulting in a change of the second amino acid in the signal peptide from glycine (G)(G<u>G</u>A) to glutamic acid (E)(G<u>A</u>A).

RESULTS

pMFG-hFVIIIΔB expression vectors

Two different B domain deletion versions of the human FVIII cDNA were introduced into MFG retroviral vector plasmid DNA as described in Materials and Methods. Figure 1 shows encoded B domain-deleted versions of hFVIII used in our studies. Protein molecules $hFVIII_G\Delta B_{814}$ and $hFVIII_E\Delta B_{814}$ (Fig. 1A and B) lack 814 aa (aa 747 to 1560) of the B domain but retain all critical thrombin cleavage sites in addition to the proteolytic site at aa 1648 involved in the initial processing of hFVIII (Kaufman, 1992) The pMFG-$hFVIII_G\Delta B_{814}$ plasmid contains the engineered Kozak translation sequence GCC(ATG)G (Kozak, 1989) and therefore encodes a glycine (G) in amino acid position 2 of the signal peptide of $hFVIII_G\Delta B_{814}$.

To determine whether a further optimized Kozak sequence would improve expression in host cells, the pMFG-$hFVIII_G\Delta B_{814}$ Kozak sequence was modified to create pMFG-$hFVIII_E\Delta B_{814}$ by substitution of the G nucleotide with an A at position +5 to match an extended optimized Kozak consensus sequence GCC(ATG)GA (Grünert and Jackson, 1994). The resulting nucleotide switch converts the second amino acid of the signal peptide from a glycine (G) to a glutamic acid (E). The third hFVIII plasmid, pMFG-$hFVIII_E\Delta B_{906}$, also contains the same Kozak-like sequence as that found in pMFG-$hFVIII_E\Delta B_{814}$ but encodes a larger 906-aa deletion (aa 743 to 1648) of the B domain, including the proteolytic site at aa 1648. Like $hFVIII_G\Delta B_{814}$ and $hFVIII_E\Delta B_{814}$, protein molecule $hFVIII_E\Delta B_{906}$ also retains all sites required for thrombin activation.

Canine bone marrow stromal cells secrete biologically active hFVIII in vitro *following transfection with MFG-hFVIII plasmid vectors*

To test whether BMSCs are appropriate host cells for the secretion of biologically active FVIII, canine BMSCs grown in 25-cm^2 tissue culture flasks were transfected with either pMFG-$hFVIII_G\Delta B_{814}$, pMFG-$hFVIII_E\Delta B_{814}$, or pMFG-$hFVIII_E\Delta B_{906}$ plasmid vectors. Four days after transfection, culture media conditioned for 24 hr were harvested and procoagulant activity assayed by the one-stage coagulation assay for hFVIII:c. BMSCs individually transfected with plasmid DNA from all three constructs secreted functionally active hFVIII:c protein at maximal levels ranging from 280 to 386 mU/10^6 cells/24 hr, with average levels of 202 to 230 mU/10^6 cells/24 hr (Table 1). The hFVIII procoagulant activity levels secreted by BMSCs transfected by the three hFVIII plasmid vectors in multiple transfection experiments were not significantly different ($p > 0.05$). These data indicate that the changes to the engineered Kozak-like sequence did not significantly alter expression ($p > 0.05$).

To confirm that detected hFVIII:c procoagulant activities secreted by BMSCs transfected with either pMFG-$hFVIII_E\Delta B_{814}$ or pMFG-$hFVIII_E\Delta B_{906}$ were indeed hFVIII:c derived, conditioned media of similar procoagulant activities were incubated with either specific hFVIII:c-inhibitory or nonspecific antibodies or buffer alone prior to subsequent activity assays. Conditioned media from mock-transfected BMSC controls incubated with the same series of antibodies were used as background controls. Prior to incubation, the $hFVIII_E\Delta B_{814}$ and $hFVIII_E\Delta B_{906}$ samples exhibited procoagulant activities of 350 and 400 mU/ml, respectively. Human normal pooled plasma (NP), adjusted to similar initial activity, was used as a positive control.

TABLE 1. *In Vitro* EXPRESSION OF BIOLOGICALLLY ACTIVE hFVIII FROM BMSCs TRANSFECTED WITH pMFG-hFVIIIΔB PLASMID VECTORS, OR TRANSDUCED WITH pMFG-hFVIIIΔB RETROVIRAL VECTORS GENERATED BY PRODUCER CLONES[a]

Vector	*Number of samples* (n)	*hFVIII activity average ± SD* (*mU/10⁶ cells/24 hr*)
Plasmid vectors		
pMFG-$hFVIII_G\Delta B_{814}$	10	227 ± 73
pMFG-$hFVIII_E\Delta B_{814}$	6	202 ± 49
pMFG-$hFVIII_E\Delta B_{906}$	10	230 ± 83
Retroviral vectors		
MFG-$hFVIII_G\Delta B_{814}$		
Producer clone C25	20	1160 ± 795
Producer clone C46	28	1620 ± 666
MFG-$hFVIII_E\Delta B_{906}$		
Producer clone 10B6	14	1130 ± 381
Producer clone 10D10	10	1144 ± 296

[a]Canine BMSCs were transfected with plasmid vector one to three times, or transduced at least twice with retroviral vector generated from producer clones. Culture media conditioned for 24 hr were harvested and biological activity assayed by the one-stage coagulation assay for hFVIII:c. By definition, 1 unit of FVIII activity is equivalent to the activity of FVIII present in 1 ml of normal pooled human plasma and equals 100% clotting activity. Human FVIII activity is presented as average values ± SD.

When murine monoclonal IgG antibody (maHF8-ab) directed against the hFVIII light chain or sheep anti-FVIII:c polyclonal antibody (CL20035A) was incubated with human NP, FVIII:c activity was abolished to below background levels (i.e., >89% inhibition relative to incubation with buffer alone), as expected. Similarly, these antibodies also mediated a significant loss in procoagulant activity ranging between 70 and >90%, when incubated with either pMFG-hFVIII$_E\Delta B_{814}$ or pMFG-hFVIII$_E\Delta B_{906}$ hFVIII in medium conditioned by transfected BMSCs. Incubation with nonspecific murine G8523 anti-human growth hormone control IgG did not inhibit hFVIII:c activity, with resultant activity levels identical to levels when hFVIII was incubated in buffer alone. The results verify that functionally active recombinant hFVIII:c was secreted from transfected BMSCs.

Comparison of percentage of transfected BMSCs harboring pMFG-hFVIIIΔB vector DNA, expressing hFVIII, and levels of secreted hFVIII procoagulant activity

Plasmid vector-transfected BMSCs grown in duplicate sets of 25-cm^2 flasks per vector displaying similar procoagulant activities at 259, 280, and 311 mU/10^6 cells/24 hr for plasmids pMFG-hFVIII$_G\Delta B_{814}$, pMFG-hFVIII$_E\Delta B_{814}$, and pMFG-hFVIII$_E\Delta B_{906}$, respectively, were fixed and subjected either to hFVIII immunofluorescence for identification of BMSCs that expressed protein, or to FISH for detection of introduced vector DNA. Cells from one flask of each duplicate pair were directly fixed in culture flasks and subsequently analyzed by immunofluorescence. Cells were trypsinized from the remaining flasks and immediately cytospun onto multiple glass slides in preparation for examination by FISH and immunofluorescence.

Immunofluorescence analysis of transfected cells showed similarly low percentages of cells expressing hFVIII for all three vectors, ranging between 3.2 and 8.9% for cells immunostained in flasks (Fig. 2A–C), and 3.6, 7.0, and 2.9% for cytospun cells transfected with plasmids pMFG-hFVIII$_G\Delta B_{814}$, pMFG-hFVIII$_E\Delta B_{814}$, and pMFG-hFVIII$_E\Delta B_{906}$, respectively (an example of typical results is shown in Fig. 3C). These studies demonstrated similar percentages of cells expressing hFVIII between cytospun BMSCs and BMSCs fixed in flasks. No immunofluorescent staining was evident in mock-transfected samples (Figs. 2D and 3D) or plasmid-transfected samples stained with nonspecific murine IgG$_{2a}$(κ) antibody (data not shown). All individual components of these analyses were performed at least two additional times with similar results. Thus, these results demonstrated a high level of expression from a relatively low percentage of cells.

Plasmid pMFG-hFVIII$_G\Delta B_{814}$ DNA was used as a probe to visualize intracellular vector DNA by FISH, and propidium iodide was used to counterstain nuclei in transfected cells. Stromal cells transfected with the three vectors showed a similar scattering of positive hybridization signals on cells, an example of which is shown in Fig. 3. In many instances multiple hybridization signals were detected within individual cells of a plasmid-transfected cell population (Fig. 3A). Hybridization signals were largely localized to the cytoplasm, indicating that while introduction of vector into cells was efficient, the majority of the plasmid resided outside the nucleus. No hybridization signals were evident in either mock-transfected cells (Fig. 3B) or plasmid-transfected cells not exposed to probe (data not shown). Vector sequences were detected in 47.5, 42.3, and 42.0% of cells transfected with plasmids pMFG-hFVIII$_G\Delta B_{814}$, pMFG-hFVIII$_E\Delta B_{814}$, and pMFG-hFVIII$_E\Delta B_{906}$, respectively. Thus, while uptake of DNA was relatively efficient, only a small proportion of cells (2.9 to 8.9%) actually expressed hFVIII antigen.

Percent normal biological activity and specific activity of hFVIII secreted from transfected cells

The percent normal biological activity was compared with the hFVIII biological activity in pooled human plasma. To evaluate percent normal biological activity of a wide range of hFVIII procoagulant and antigen levels, BMSCs were transfected with pMFG-hFVIIIΔB plasmids in a total of 39 individual transfections. Media conditioned for 48 hr were collected from transfected BMSCs and both hFVIII clotting activity and antigen levels were determined (Table 2). The percent normal biological activity of the hFVIII was calculated by comparing the hFVIII clotting activity with the ELISA-determined hFVIII protein level, using normal pooled human plasma as the standard.

The hFVIII protein encoded by vectors pMFG-hFVIII$_G\Delta B_{814}$ and pMFG-hFVIII$_E\Delta B_{814}$ are identical except for a single amino acid difference in the second position of the signal peptide, which is not included in the mature protein following secretion (see Fig. 1). Factor VIII produced by transfection of these plasmid vectors into BMSCs were similar and exhibited

FIG. 2. Immunofluorescence detection of human FVIII in BMSCs following transfection with pMFG-hFVIIIΔB plasmid vectors or transduction with MFG-hFVIIIΔB retroviral vectors. Immunofluorescence was performed using anti-hFVIII antibody ESH 8 in conjunction with rhodamine (red) secondary antibody and a DAPI (blue) nuclear counterstain on BMSCs transfected with vector pMFG-hFVIII$_G\Delta B_{814}$ (**A**), pMFG-hFVIII$_E\Delta B_{814}$ (**B**), pMFG-hFVIII$_E\Delta B_{906}$ (**C**), and mock transfected cells (**D**) or on BMSCs transduced with C46 for MFG-hFVIII$_G\Delta B_{814}$ (**E**) and 10D10 for MFG-hFVIII$_E\Delta B_{906}$ (**F**). A total of 8.9, 7.9, 3.2, 0, 73, and 68% cells expressed hFVIII in (**A**–**F**), respectively. Original magnification: ×400.

FIG. 3. Fluorescence *in situ* hybridization (FISH) detection of BMSCs harboring plasmid vector DNA and immunofluorescence detection of hFVIII expression in BMSCs following transfection with pMFG-hFVIII$_G\Delta B_{814}$. BMSCs transfected with pMFG-hFVIII$_G\Delta B_{814}$ (**A** and **C**) or mock-transfected cells (**B** and **D**) were harvested 3 days following transfection and cytospun (see Materials and Methods). FISH analysis was performed using a fluorescein-labeled probe (yellow) and a propidium iodide (red) nuclear counterstain (**A** and **B**). A total of 47.5% of transfected cells harbor plasmid vector. Immunofluorescence was performed using anti-hFVIII antibody ESH 8 in conjunction with a rhodamine (red) secondary antibody and a DAPI (blue) nuclear counterstain (**C** and **D**). A total of 3.6% of transfected cells expressed hFVIII protein. Original magnification: ×400.

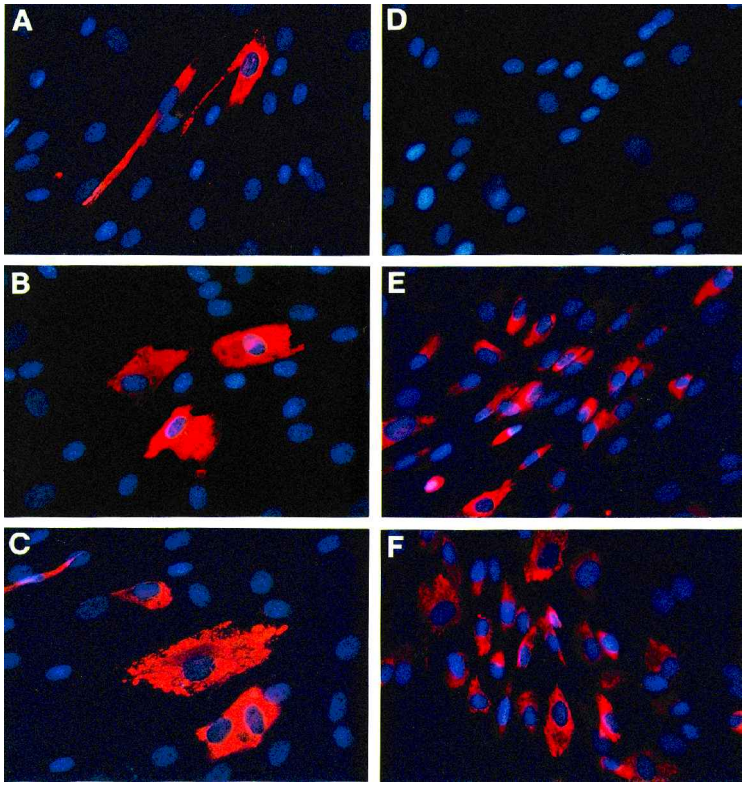

FIG. 2.

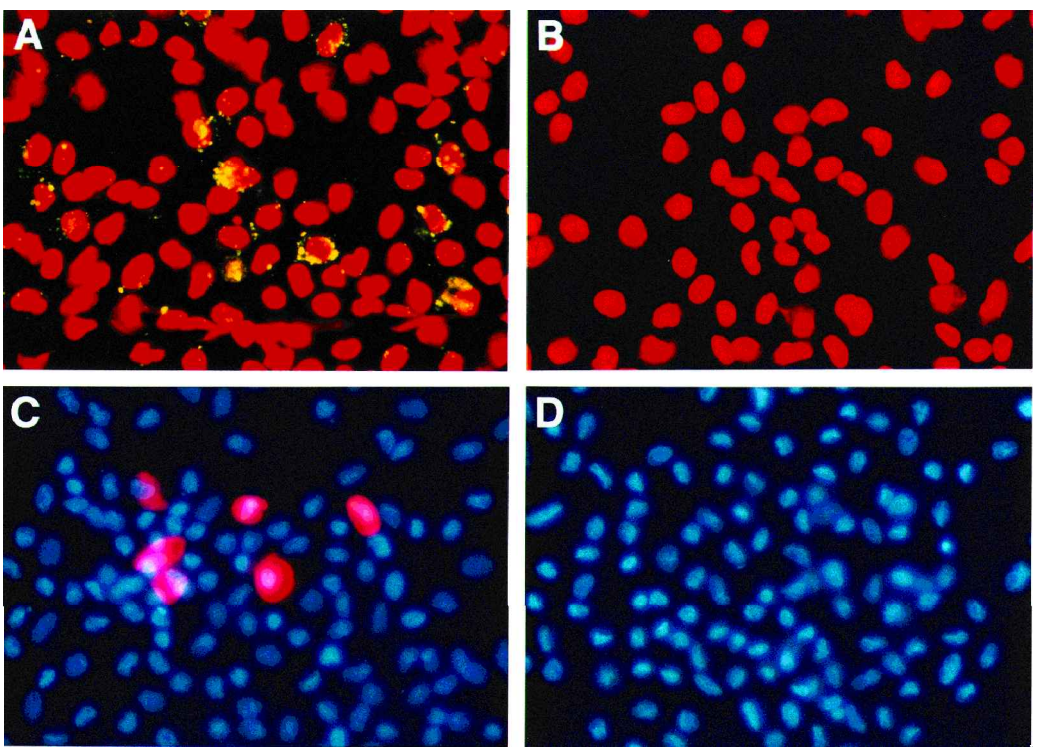

FIG. 3.

a high percentage of normal biological activities—more than 148.5% for a range of expressed procoagulant and antigen levels. The specific activities of these hFVIII species ranged from an average of 7424 to 8220 U/mg. In contrast, transfection with pMFG-hFVIII$_E\Delta B_{906}$ (which includes the deletion of the position 1648 proteolytic site that is retained in the other vectors), yielded a substantially lower percentage of normal biological active hFVIII, only 53.4%, and a correspondingly lower specific activity of 2672 U/mg. This vector also lacks an additional 92 amino acids from the B domain as compared with the other vectors. Therefore, the disparity in percent normal biological activity between vectors may be explained by structural differences in secreted protein forms.

Western analysis of secreted hFVIII from plasmid vector-transfected BMSCs

To characterize and compare the secreted forms of hFVIII-ΔB expressed by BMSCs transfected with the different plasmid vectors, immunoprecipitation followed by Western blot analyses were performed. Clinical-grade full-length recombinant hFVIII (rAHF) (Bioclate; Centeon Pharmaceuticals, Kankakee, IL) was diluted in conditioned media from mock-transfected cells and used as a positive control. Conditioned media from mock-transfected cells were used as a negative control.

Figure 4A shows a Western analysis using hFVIII light chain-specific maHF8c-ab as the detecting antibody. The majority of hFVIII$_G\Delta B_{814}$ and hFVIII$_E\Delta B_{814}$, containing the position 1648 proteolytic site located at the B-domain C terminus, was effectively processed to the 79/80-kDa light chain doublet (Fig. 4A, lanes 2 and 4) consistent with the 79/80-kDa light chain doublet seen with rAHF (Fig. 4A, lane 1). Significantly, the 80-kDa band was more prominent than the 79-kDa band. The faint 185-kDa band represented the small fraction of still unprocessed single contiguous heavy–light chain (Fig. 4A, lanes 2 and 4). As the maHF8c-ab detecting antibody is specific for the light chain, the heavy chain was not detected, as expected. In contrast, hFVIII$_E\Delta B_{906}$, which does not contain the position 1648 proteolytic site, remained largely unprocessed and appeared predominantly as a single heavy–light chain 175-kDa band (Fig. 4A, lane 6). Some processing of hFVIII$_E\Delta B_{906}$ to a 79-kDa light chain band was detected (Fig. 4A, lane 6). Western analysis utilizing a polyclonal sheep anti-hFVIII antibody capable of detecting both hFVIII heavy and light chains confirmed and expanded on these observations (Fig. 4B). The hFVIII polyclonal antibody resulted in considerable nonspecific binding to fetal calf serum proteins in the tissue culture medium in approximately the 85- to 170-kDa region, as demonstrated by the rAHF spiking experiments (Fig. 4B, lanes 1–4) and the mock transfection of BMSC analyses (Fig. 4B, lanes 9 and 10). The nonspecific background makes it difficult to identify unequivocally the >90-kDa heavy chain band of expressed hFVIII. In agreement with previous reports rAHF is predominantly composed of the heavy chain (200 kDa), which includes the B domain, and the light chain (79/80 kDa) with minimal quantities of processed intermediates (Fig. 4B, lanes 1 and 3) (Adamson, 1994). The 80-kDa light chain fragment appeared to be more prominent than the 79-kDa fragment. The hFVIII$_G\Delta B_{814}$ was expressed as a processed heterodimer as demonstrated by the lack of detection of a 185-kDa heavy–light chain monomer and by the presence of the 79/80-kDa light chain (Fig. 4B, lane 5). Here, too, the 80-kDa fragment appeared more prominent than the 79-kDa fragment. In contrast, the hFVIII$_E\Delta B_{906}$ protein was expressed as a single heavy–light chain monomer with some proteolysis as demonstrated by the presence of 79/80-kDa light chain (Fig. 4B, lane 7). No hFVIII peptides were detected in mock controls (Fig. 4A, lanes 8 and 9; Fig. 4B, lanes 9 and 10).

To determine whether hFVIII molecules secreted by transfected BMSCs were correctly cleaved to peptides of the predicted size on thrombin treatment, immunoprecipitated samples were incubated with thrombin for 2 min at 37°C prior to SDS-

Table 2. Percent Normal Biological Activity, and Specific Activity, of Human Factor VIII Expressed and Secreted *in Vitro* by BMSCs Transfected with pMFG-hFVIIIΔB Plasmid Vectors, or Transduced with MFG-hFVIIIΔB Retroviral Vectors[a]

Vector	*Number of samples* (n)	*Percent normal biological activity*	*Specific activity (U/mg)*
Plasmid vectors			
pMFG-hFVIII$_G\Delta B_{814}$	15	164.4 ± 26.7	8220 ± 1333
pMFG-hFVIII$_E\Delta B_{814}$	7	148.5 ± 28.7	7424 ± 1433
pMFG-hFVIII$_E\Delta B_{906}$	17	53.4 ± 13.1	2672 ± 654
Retroviral vectors			
MFG-hFVIII$_G\Delta B_{814}$			
Producer clone C25	10	94.5 ± 22.4	4727 ± 1122
Producer clone C46	24	92.9 ± 19.8	4602 ± 991
MFG-hFVIII$_E\Delta B_{906}$			
Producer clone 10B6	7	52.5 ± 18.8	2625 ± 939
Producer clone 10D10	9	38.2 ± 6.8	1910 ± 340

[a]Canine BMSCs were transfected with plasmid vector two to three times, and samples analyzed from media conditioned for 48 hr. BMSCs were transduced at least twice with retroviral vector generated from producer clones, and media conditioned for 24 hr were harvested for analysis. The percent normal biological activity of hFVIII in conditioned medium was calculated as the percent biological activity of the sample relative to normal pooled plasma (100%) and adjusting for concentration of hFVIII in the sample, as determined by ELISA, using normal pooled human plasma as standard (200 ng/ml). Percent normal biological activity and specific activity are expressed as average values ± SD.

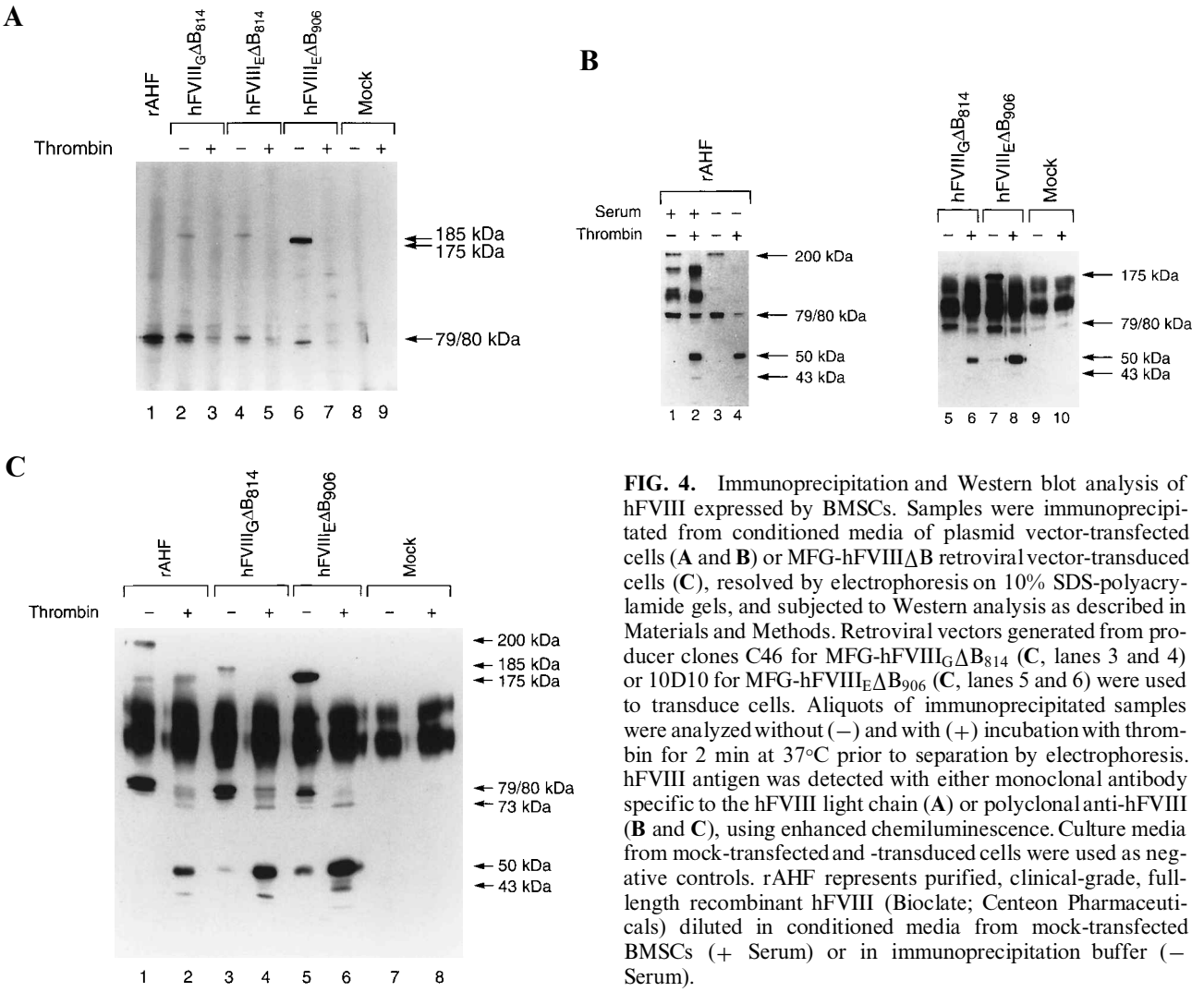

FIG. 4. Immunoprecipitation and Western blot analysis of hFVIII expressed by BMSCs. Samples were immunoprecipitated from conditioned media of plasmid vector-transfected cells (**A** and **B**) or MFG-hFVIIIΔB retroviral vector-transduced cells (**C**), resolved by electrophoresis on 10% SDS-polyacrylamide gels, and subjected to Western analysis as described in Materials and Methods. Retroviral vectors generated from producer clones C46 for MFG-hFVIII$_G$ΔB$_{814}$ (**C**, lanes 3 and 4) or 10D10 for MFG-hFVIII$_E$ΔB$_{906}$ (**C**, lanes 5 and 6) were used to transduce cells. Aliquots of immunoprecipitated samples were analyzed without (−) and with (+) incubation with thrombin for 2 min at 37°C prior to separation by electrophoresis. hFVIII antigen was detected with either monoclonal antibody specific to the hFVIII light chain (**A**) or polyclonal anti-hFVIII (**B** and **C**), using enhanced chemiluminescence. Culture media from mock-transfected and -transduced cells were used as negative controls. rAHF represents purified, clinical-grade, full-length recombinant hFVIII (Bioclate; Centeon Pharmaceuticals) diluted in conditioned media from mock-transfected BMSCs (+ Serum) or in immunoprecipitation buffer (− Serum).

PAGE separation and subsequent Western analyses. Thrombin treatment caused the single heavy–light chain (185/175-kDa) monomer to disappear and the 79/80-kDa light chain to diminish significantly in intensity for hFVIII products of all three vectors (Fig. 4A, lanes 3, 5, and 7; Fig. 4B, lanes 6 and 8). The expected 50- and 43-kDa fragments derived from cleavage of the heavy chain and detected with the polyclonal antibody were visible after thrombin treatment of all samples as well as in rAHF samples (Fig. 4B, lanes 2, 4, 6, and 8). The predicted thrombin-generated 73-kDa light chain fragment, however, could not be detected by either antibody owing to the apparent weak immunoreactivity toward the 73-kDa thrombin-cleaved fragment (see below).

Expression of hFVIII by canine BMSCs transduced with MFG-hFVIIIΔB retroviral vectors

Having established BMSCs as appropriate host cells for the delivery of biologically active FVIII by transient transfection studies, we next generated amphotropic packaging cell lines producing retroviral vectors to improve levels of expression from BMSCs. We chose to isolate producer clones only from MFG-hFVIII$_G$ΔB$_{814}$ and MFG-hFVIII$_E$ΔB$_{906}$, since transfection experiments established that vector pMFG-hFVIII$_E$ΔB$_{814}$ did not produce hFVIII at levels greater than that of pMFG-hFVIII$_G$ΔB$_{814}$.

MFG-hFVIII$_G$ΔB$_{814}$ producer clones C25 and C46, and MFG-hFVIII$_E$ΔB$_{906}$ producer clones 10B6 and 10D10, were generated and viral supernatant harvested as described in Materials and Methods. BMSCs were subjected to at least two rounds of transduction. Culture media conditioned for 24 hr by transduced cells were analyzed using hFVIII procoagulant assays and demonstrated markedly higher levels of hFVIII activity (1130 to 1620 mU/10^6 cells/24 hr) than that obtained by plasmid transfection (Table 1). Transduced cells expressed maximal levels, which ranged from 1851 to 4858 mU/10^6 cells/24 hr. BMSCs expressed similar hFVIII clotting activity levels ($p > 0.05$) following transduction by vector from all four producer clones representing the two different MFG-hFVIIIΔB vector forms.

Immunofluorescence detection of hFVIII in transduced BMSCs demonstrated that the majority of cells expressed pro-

tein after only two rounds of transduction by C46 for MFG-hFVIII$_G\Delta B_{814}$ or 10D10 for MFG-hFVIII$_E\Delta B_{906}$ (Fig. 2E and F). In the experiment represented in Fig. 2, BMSC transduction by C46 resulted in 73% hFVIII-expressing cells yielding 1262 mU/10^6 cells/24 hr, and transduction by 10D10 similarly resulted in 68% hFVIII-expressing cells yielding 971 mU/10^6 cells/24 hr. No immunofluorescent staining was evident in mock-transduced cells or transduced cells stained with a non-specific murine $IgG_{2a}(\kappa)$ negative control antibody (data not shown).

Percent normal biological activity and specific activity of secreted hFVIII from transduced cells

Percent normal biological activity was evaluated from 50 individual BMSC transductions representing a wide range of procoagulant and antigen levels. Analysis of culture media, conditioned for 24 hr from BMSCs transduced by viral supernatant from the four producer clones revealed the same trend ascribed to plasmid vectors pMFG-hFVIII$_G\Delta B_{814}$ and pMFG-hFVIII$_E\Delta B_{906}$ as previously found for transfected BMSCs (Table 2). Retroviral vector MFG-hFVIII$_G\Delta B_{814}$ derived from producer clones C25 and C46 supported BMSC expression of hFVIII with more than 92.9% normal biological activities. By contrast, retroviral vector MFG-hFVIII$_E\Delta B_{906}$-derived producer clones 10B6 and 10D10 resulted in hFVIII expression from transduced BMSCs with significantly lower percentages of normal biological activity, with averages of 54.6 and 38.2%, respectively. As previously noted, vectors from all four clones supported expression of statistically similar functional hFVIII clotting activity (mU/10^6/24 hr) from transduced BMSCs. However, the average hFVIII$_E\Delta B_{906}$ protein expression levels of 511.1 ng/10^6 cells/24 hr (10B6) and 451.2 ng/10^6 cells/24 hr (10D10) were much higher than the average hFVIII$_G\Delta B_{814}$ protein expression levels of 133.1 ng/10^6 cells/24 hr (C25) and 285.7 ng/10^6 cells/24 hr (C46) (data not shown). The percent normal biological activity of hFVIII$_E\Delta B_{906}$ was consistently lower than that of hFVIII$_G\Delta B_{814}$, even when equivalent protein expression levels (at high or low levels) were evaluated (data not shown). Thus, while hFVIII$_E\Delta B_{906}$ was generally secreted at high levels its activity was as much as 45.4 to 61.8% less than that of native hFVIII in human plasma. The differences in percent normal biological activities are also reflected in differences in specific activities, with the specific activity of hFVIII$_G\Delta B_{814}$ exhibiting averages of 4602 to 4727 U/mg whereas the average specific activity of hFVIII$_E\Delta B_{906}$ was only 1910 to 2625 U/mg.

Western analysis of secreted hFVIII from retroviral vector-transduced BMSCs

Western blot analysis of hFVIIIΔB produced from BMSCs transduced with retroviral vectors from C46 and 10D10 producer lines (Fig. 4C) was consistent with the characterization of hFVIII$_G\Delta B_{814}$ and hFVIII$_E\Delta B_{906}$ secreted from plasmid vector-transfected cells (Fig. 4A and B). Analysis using polyclonal anti-hFVIII detecting antibody confirmed secretion of hFVIII$_G\Delta B_{814}$ as mostly heterodimeric heavy and light chains, as shown by the predominant 79/80-kDa light chain bands, with the 80-kDa band being more prominent (Fig. 4C, lane 3). The non-specific bands obscure the >90-kDa heavy chain. This is in contrast to hFVIII$_E\Delta B_{906}$ secretion as mostly single 175-kDa heavy–light monomers (Fig. 4C, lane 5). A minor fraction of secreted hFVIII$_G\Delta B_{814}$ and hFVIII$_E\Delta B_{906}$, accumulated in conditioned media for 24 hr, also appeared to be cleaved to 50-kDa heavy chain-derived fragments (Fig. 4C, lanes 3 and 5). All correctly sized fragments were generated and clearly visible on thrombin treatment, including the predicted 50- and 43-kDa heavy chain-derived products and the 73-kDa light chain (Fig. 4C, lanes 4 and 6), consistent with identical bands derived from thrombin treatment of rAHF (Fig. 4C, lane 2).

Thrombin activation profiles of hFVIII expressed by BMSCs

We examined the kinetics of thrombin activation of hFVIII expressed into conditioned media by BMSCs transduced with MFG-hFVIII$_G\Delta B_{814}$, and MFG-hFVIII$_E\Delta B_{906}$ retroviral vectors. These samples, as well as normal pooled plasma, were adjusted to similar procoagulant activities using conditioned media from mock-transduced cells as a diluent, then incubated with human thrombin (300 mU/ml) at 37°C. At specified time intervals aliquots were removed and immediately subjected to one-stage clotting assays (Fig. 5). Activation profiles of expressed hFVIII from cells transduced by the two vectors were similar and exhibited the expected biphasic curves, similar to that seen with plasma hFVIII. In all cases, activities reached a peak at 2 min and were followed by a subsequent decay. These results further demonstrate that hFVIII expressed from BMSCs maintains intact thrombin sites that are capable of becoming functionally activated in a manner similar to that of native FVIII.

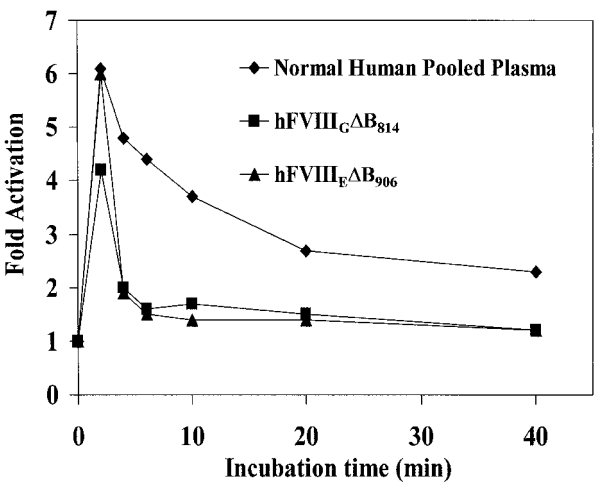

FIG. 5. Thrombin activation profiles of biologically active hFVIII expressed by MFG-hFVIIIΔB-transduced BMSCs. Conditioned media from BMSCs transduced with C46 for MFG-hFVIII$_G\Delta B_{814}$ or with 10D10 for MFG-hFVIII$_E\Delta B_{906}$ were adjusted to approximately equal procoagulant activity (700 mU/ml) and incubated with human thrombin (300 mU/ml) at 37°C. At the indicated time intervals aliquots were removed, and immediately assayed in a one-stage clotting assay. Normal pooled plasma was used to generate standard curves. The change in coagulant activity expressed as fold activation was plotted as a function of incubation time with thrombin. Represented here are typical results obtained from one of multiple experiments.

DISCUSSION

We have previously demonstrated delivery of transient therapeutic levels of blood coagulation factor IX to the systemic circulation of dogs by *ex vivo* retroviral vector-modified BMSCs in the development of the BMSC system for gene therapy of hemophilia B (Cherington *et al.*, 1998; Chiang *et al.*, 1998). In our current study, we demonstrate high-level expression of biologically active hFVIII from retroviral vector-transduced BMSCs (1000–4000 mU/10^6 cells/24 hr), with the intention of broadening the use of these cells in an *ex vivo* gene therapy for hemophilia A. We also demonstrate that the specific structure of B domain-deleted variants determines their biological activities.

A target cell type for the *ex vivo* gene therapy of hemophilia A must possess the capacity for correct posttranslational modification and complex processing of hFVIII for high-level production of optimally functional protein (Pavirani *et al.*, 1987). Prior investigations have demonstrated that many cell types were either unsuitable for the production of hFVIII, or produce inadequately low levels of hFVIII (Hoeben *et al.*, 1990; Israel and Kaufman, 1990; Lynch *et al.*, 1993; Chuah *et al.*, 1995). In the current study, we have cloned two different B domain-deleted forms of hFVIII into the efficient MFG retroviral vector (Dranoff *et al.*, 1993) for comparison of expression rates and structural properties of secreted hFVIII in canine BMSCs. One form, within two vectors differing in the Kozak translational initiation sequences and therefore the second amino acid of the signal peptide (hFVIII$_G\Delta B_{814}$/hFVIII$_E\Delta B_{814}$), encodes a deletion of 814 aa (aa 747–1560) but retains the native proteolytic site at aa 1648 involved in the cleavage of the heavy from the light chain (Pittman and Kaufman, 1988). The other form (hFVIII$_E\Delta B_{906}$) encodes a deletion of 906 aa (aa 743–1648) and does not retain the aa 1648 proteolytic site. We conducted plasmid transfection studies to evaluate rapidly whether BMSCs are appropriate host cells for hFVIII production and established that BMSCs are indeed capable of high-level procoagulant hFVIII activities (secreting as much as 386 mU/10^6 cells/24 hr). All three vectors supported expression of statistically similar levels of hFVIII procoagulant activity from transfected BMSCs. Only a low percentage (2.9 to 8.9%) of these cells expressed hFVIII, even though 42 to 47.5% harbored plasmid vector (predominantly located in the cytoplasm as opposed to the nuclei). These results are consistent with other studies with primary human fibroblasts (Coonrod *et al.*, 1997), as well as a number of cell lines in which the major impedance to effective gene delivery is nuclear translocation of the plasmid vector (Labat-Moleur *et al.*, 1996).

We assessed the effectiveness of further optimizing the Kozak sequence by inclusion of nucleotide A at position +5, to improve translation initiation (Grünert and Jackson, 1994). Parallel comparison of hFVIII produced by BMSCs transfected by plasmid vector pMFG-hFVIII$_G\Delta B_{814}$ or pMFG-hFVIII$_E\Delta B_{814}$ showed no differences in FVIII procoagulant activities. These forms of hFVIII contain the same B domain deletion (aa 747–1560) but differ in the +5 nucleotide position, which affects only the second amino acid of the secretory signal sequence. The further optimized Kozak sequence did not improve expression beyond that of the less optimized Kozak sequence. In addition, Sarver *et al.* (1987) reported no differences in expressed activity when full-length or B domain-deleted FVIII, both modified to contain an ATG consensus sequence, were compared with equivalent unmodified forms containing the native translation initiation sequence.

Despite the low percentage of transfected BMSCs expressing hFVIII, secreted levels were comparable to those previously reported for retroviral vector transduction of a variety of cell types (100 to 300 mU/10^6 cells/24 hr) from which FVIII was quantified only after selectable marker amplification of transduced cells (Hoeben *et al.*, 1990; Israel and Kaufman, 1990; Lynch *et al.*, 1993; Chuah *et al.*, 1995). Our current FVIII levels were achieved by plasmid transfection without the need for gene-modified cell enrichment. One report suggested that MFG retroviral vectors possess greater levels of spliced mRNA per vector copy number when compared with other vectors, thus making it a superior vector when high-level production is desired in target cells (Krall *et al.*, 1996). This may explain why significant secreted activity was achieved even when the MFG vector was utilized as a plasmid expression vector in our studies.

Vector MFG-hFVIII$_E\Delta B_{906}$ lacks the native position 1648 proteolytic site, and as a result BMSCs, transfected and transduced with this vector, expressed predominantly unprocessed 175-kDa contiguous single heavy–light chains. Some processing did occur as evidenced by the presence of 79-kDa light chains, most likely owing to a previously identified minor proteolysis site located at the start of the A3 domain (aa 1658) (Lind *et al.*, 1995). In contrast, vectors MFG-hFVIII$_G\Delta B_{814}$ and MFG-hFVIII$_E\Delta B_{814}$, which retain the position 1648 proteolytic site, express hFVIII molecules that are effectively cleaved to heterodimers composed of 79/80-kDa light and >90-kDa heavy chains while only a minor fraction is secreted as full-length 185-kDa monomers. The prominence of the 80-kDa over the 79-kDa light chain fragments of hFVIII expressed by cells transfected or transduced by vectors retaining the native position 1648 proteolytic site, similarly observed in the recombinant full-length hFVIII product (rAHF), suggests that the position 1648 proteolytic site is preferentially utilized over the minor proteolytic site whether or not the B domain is intact or largely deleted. These Ca^{2+} abridged heavy and light chain heterodimers are expected to be similar to heterodimers of heavy chains (heterogeneously sized in plasma) and light chains isolated from plasma (Eaton *et al.*, 1986a). Even so, peptide chains derived from different vector forms, and secreted as either heterodimers or monomers, retained functionally intact thrombin sites as evidenced by release of specific heavy chain-derived and light chain-derived peptides on thrombin treatment. In all cases, results were consistent between transfected and transduced BMSCs. Functional thrombin sites were confirmed by the similarity of thrombin activation profiles of the heterodimeric and monomeric forms of recombinant FVIII relative to that of plasma-derived hFVIII.

On the basis of the criterion of rate of production of hFVIII procoagulant activity, statistically similar ($p > 0.05$) high levels of the three forms of hFVIII, hFVIII$_E\Delta B_{906}$ and hFVIII$_G\Delta B_{814}$/hFVIII$_E\Delta B_{814}$, were expressed from transfected and transduced BMSCs. However, in applying hFVIII as a therapeutic protein in a clinical setting it is important to compare biological activity levels of secreted B domain-deleted forms with that expected of native plasma hFVIII. By comparing secreted

hFVIII antigen levels (quantified by ELISA) with associated procoagulant activities it was possible to estimate levels of biological activity of total expressed hFVIII protein relative to that of native plasma hFVIII. Human FVIII expressed as $hFVIII_E\Delta B_{906}$ monomers consistently exhibited only ~50% biological activity when expressed by either plasmid transfection or by retroviral vector transduction. The $hFVIII_E\Delta B_{906}$ antigen levels secreted by BMSCs following transduction of 10D10 and 10B6 retroviral vector producer clones were as high as 757 ng/10^6 cells/24 hr. In contrast, the biological activity of expressed MFG-$hFVIII_E\Delta B_{814}$ hFVIII was high (164.4 and 94.5% for hFVIII expressed by transfected and transduced BMSCs, respectively). Specific activity for $hFVIII_G\Delta E_{906}$ ranged from 1910 to 2672 U/mg whereas specific activity for $hFVIII_G\Delta B_{814}$ ranged from 4602 to 8220 U/mg. Specific activities for these B domain-deletion forms may be underestimated since ELISA-determined hFVIII protein levels were extrapolated from standard curves of human normal plasma. The hFVIII in human normal plasma is larger and heterogeneously sized, owing to variable proteolysis of the B domain. The specific activity of $hFVIII_G\Delta B_{814}$ and $hFVIII_E\Delta B_{814}$ is comparable to the specific activity of 4000 to 7000 U/mg typical of highly purified hFVIII recombinant products (Berntorp, 1997). The biological activities we obtained with MFG-$hFVIII_E\Delta B_{906}$ are in agreement with previously reported 50–70% biological activities obtained after transduction of human myoblasts, endothelial cells, and skin fibroblasts using an MFG-based vector identical to MFG-$hFVIII_E\Delta B_{906}$, which also resulted in high hFVIII antigen expression levels (1000–2000 ng/10^6 cells/24 hr) (Dwarki *et al.*, 1995).

The findings here suggest that $hFVIII_G\Delta B_{814}$/$hFVIII_E\Delta B_{814}$ molecules would be more favorable for gene therapy than $hFVIII_E\Delta B_{906}$, based on potential 100% biological activity, high specific activity, and its heterodimeric structural similarity to native plasma hFVIII. Moreover, it has been shown in a clinical trial that patient administration of r-VIII SQ, a recombinant hFVIII B domain-deletion product lacking aa 743–1638 and expressed in heterodimeric forms, was a safe, well-tolerated, and efficacious drug (Berntorp, 1997). Protein $hFVIII_E\Delta B_{906}$ may not be appropriate as a gene therapy product since 50% or more is likely to be inactive, necessitating the administration of greater levels of antigen to achieve therapeutic procoagulant levels. Excess circulatory levels of recombinant hFVIIIΔB may increase the risk of inhibitor development, a serious complication of hFVIII gene replacement therapy for hemophilia A (Hoyer, 1995). Furthermore, the presence of a large proportion of apparently defective $hFVIII_E\Delta B_{906}$ is highly undesirable. *In vivo* experiments would be required to determine the actual pharmacokinetic fates of these different B domain-deleted forms of hFVIII.

We speculate that the higher $hFVIII_G\Delta B_{814}$/$hFVIII_E\Delta B_{814}$ percent biological activities are due to their expression as heavy–light chain heterodimers with closer structural similarity to the heterodimeric native forms largely bound in plasma (Eaton *et al.*, 1986a). In contrast, the $hFVIII_E\Delta B_{906}$ monomeric form is clearly structurally dissimilar from native plasma hFVIII heterodimeric forms by nature of its nonnative chain configuration. Normally, the heavy and light chains are held together in dimers by divalent Ca^{2+} bridges (Kaufman, 1992). As such, the heavy–light single chains may present a tertiary structure dissimilar to that of heavy–light chain heterodimers, owing to potential physical constraints that impair normal Ca^{2+} bridge associations between the heavy and light chain regions of the single chain. The potential for the development of hFVIII neoantigens owing to such a single-chain tertiary structure needs to be considered prior to clinical evaluation, especially in light of its limited biological activity. In addition, since the single heavy–light chain does not present a free light chain amino terminus, it is unclear how this would affect normal binding to von Willebrand factor (vWF) in light of the present data suggesting that the amino-terminal region of the light chain and carboxyl-terminal region of the heavy chain are in close proximity, forming a high-affinity binding site for vWF (Vlot *et al.*, 1998).

Chuah *et al.* (1998) also reported success in using MFG-based vector to high hFVIIIΔB expression levels (400–900 ng/10^6 cells/24 hr) by retroviral transduction of BMSCs derived from human or mouse. These high levels reported were estimated by quantification of activity units by hFVIII chromogenic assays, which were then converted to rates of antigen production using a known human plasma purified standard and assuming 1 unit equivalency to 200 ng of hFVIII per milliliter. Therefore, expression values were calculated assuming 100% normal biological activities. The authors used an MFG vector that was identical to MFG-$hFVIII_E\Delta B_{906}$ (single chain), which according to our studies produce only ~50% biologically active hFVIII. Consequently, on the basis of our results it is possible that levels of hFVIII secreted in the Chuah *et al.* study were actually much higher than those reported but that their secreted hFVIII was only partially active.

To convert a severe case of hemophilia to a mild case, it has been estimated that in humans an *in vivo* production rate of 42 μg/24 hr would be sufficient (Chuah *et al.*, 1998). This would equate to infusions of ~0.5 to 2×10^8 BMSCs (assuming 1000–4000 mU/10^6 cells/24 hr). Our previous experience with autologous infusions of *ex vivo* genetically modified BMSCs of up to 3.35×10^8 BMSCs into canine large animal models showed systemic circulation of transient human therapeutic levels of hFIX and persistence of infused BMSCs for 6 months (Cherington *et al.*, 1998; Chiang *et al.*, 1998). On the basis of our current studies on hFVIII production in BMSCs and our previous BMSC investigations in dogs, our next goal will be to determine hFVIII expression and function in hemophiliac canine models, with the intention of developing an effective gene therapy for patients with hemophilia A.

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REFERENCES

ADAMSON, R. (1994). Design and operation of a recombinant mammalian cell manufacturing process for rFVIII. Ann. Hematol. **68,** S9–S14.

ALEDORT, L.M., and BOHN, R.L. (1996). Prophylaxis and continuous infusion for hemophilia: Can we afford it? Blood Coagul. Fibrinol. **7**(Suppl. 1), S35–S37.

ANDERSSON, L.O., FORSMAN, N., HUANG, K., LARSEN, K., LUNDIN, A., PAVLU, B., SANDBERG, H., SEWERIN, K., and SMART, J. (1986). Isolation and characterization of human factor VIII: Molecular forms in commercial factor VIII concentrate, cryoprecipitate, and plasma. Proc. Natl. Acad. Sci. U.S.A. **83,** 2979–2983.

BERNTORP, E. (1997). Second generation, B-domain deleted recombinant factor VIII. Thromb. Haemost. **78,** 256–260.

BIHOREAU, N., PAOLANTONACCI, P., BARDELLE, C., FONTAINE-AUPART, M.P., KRISHNAN, S., YON, J., and ROMET-LEMONNE, J.L. (1991). Structural and functional characterization of factor VIII-delta II, a new recombinant factor VIII lacking most of the B-domain. Biochem. J. **277,** 23–31.

BOULIKAS, T. (1996). Gene therapy to human diseases: *Ex vivo* and *in vivo* studies. Int. J. Oncol. **9,** 1239–1251. [Review]

BRETTLER, D.B., and LEVINE, P.H. (1994). Clinical manifestations and therapy of inherited coagulation factor deficiencies. In *Hemostasis and Thrombosis. Basic Principles and Clinical Practice,* 3rd Ed. R.W. Colman, J. Hirsh, V.J. Marder, and E.W. Salzman, eds. (Lippincott, Philadelphia, PA) pp. 169–183.

CHERINGTON, V., CHIANG, G.G., McGRATH, C.A., GAFFNEY, A., GALANOPOULOS, T., BIZINKAUSKAS, C.B., HANSEN, M., SOBOLEWSKI, J., LEVINE, P.H., GREENBERGER, J.S., and HURWITZ, D.R. (1998). Retroviral vector modified bone marrow stromal cells secrete biologically active factor IX *in vitro* and deliver therapeutic levels of factor IX to the plasma of dogs following infusion. Hum. Gene Ther. **9,** 1397–1407.

CHIANG, G.G., McGRATH, C.A., NATALE, A., GALANOPOULOS, T., CHERINGTON, V., BIZINKAUSKAS, C.B., MERRILL, W., HANSEN, M., LEVINE, P.H., GREENBERGER, J.S., and HURWITZ, D.R. (1998). Retroviral vector transduced autologous bone marrow stromal cells express human growth hormone or human factor IX into the circulation of reinfused dogs. In *Hematopoietic Microenvironment.* A. Keating and J.S. Greenberger, eds. (Humana Press, London) (in press).

CHUAH, M.K., VANDENDRIESSCHE, T., and MORGAN, R.A. (1995). Development and analysis of retroviral vectors expressing human factor VIII as a potential gene therapy for hemophilia A. Hum. Gene Ther. **6,** 1363–1377.

CHUAH, M.K., BREMS, H., VANSLEMBROUCK, V., COLLEN, D., and VANDENDRIESSCHE, T., (1998). Bone marrow stromal cells as targets for gene therapy of hemophilia A. Hum. Gene Ther. **9,** 353–365.

CONNELLY, S., and KALEKO, M. (1997). Gene therapy for hemophilia A. Thromb. Haemost. **78,** 31–36.

COONROD, A., LI, F.-Q., and HORWITZ, M. (1997). On the mechanism of DNA transfection: Efficient gene transfer without viruses. Gene Ther. **4,** 1313–1321.

DRANOFF, G., JAFFEE, E., LAZENBY, A., GOLUMBEK, P., LEVITSKY, H., BROSE, K., JACKSON, V., HAMADA, H., PARDOLL, D., and MULLIGAN, R.C. (1993). Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proc. Natl. Acad. Sci. U.S.A. **90,** 3539–3543.

DWARKI, V.J., BELLONI, P., NIJJAR, T., SMITH, J., COUTO, L., RABIER, M., CLIFT, S., BERNS, A., and COHEN, L.K. (1995). Gene therapy for hemophilia A: Production of therapeutic levels of human factor VIII in vivo in mice. Proc. Natl. Acad. Sci. U.S.A. **92,** 1023–1027.

EATON, D., RODRIGUEZ, H., and VEHAR, G.A. (1986a). Proteolytic processing of human factor VIII. Correlation of specific cleavages by thrombin, factor Xa, and activated protein C with activation and inactivation of factor VIII coagulant activity. Biochemistry **25,** 505–512.

EATON, D.L., WOOD, W.I., EATON, D., HASS, P.E., HOLLINGSHEAD, P., WION, K., MATHER, J., LAWN, R.M., VEHAR, G.A., and GORMAN, G. (1986b). Construction and characterization of an active factor VIII variant lacking the central one-third of the molecule. Biochemistry **25,** 8343–8347.

EMAMI, S., MERRILL, W., CHERINGTON, V., CHIANG, G.G., KIRCHGESSER, M., APPEL, J.M., HANSEN, M., LEVINE, P.H., GREENBERGER, J.S., and HURWITZ, D.R. (1997). Enhanced growth of canine bone marrow stromal cell cultures in the presence of acidic fibroblast growth factor and heparin. *In Vitro* Cell Dev. Biol. Anim. **33,** 503–511.

FALLAUX, F.J., HOEBEN, R.C., and BRIET, E. (1995). State and prospects of gene therapy for the hemophilias. Thromb. Haemost. **74,** 266–273.

GREENBERGER, J.S. (1996). Methods for the use of stromal cells for therapeutic gene therapy. In *Methods in Molecular Medicine, Gene Therapy Protocols.* P. Robbins, ed. (Humana Press, Totowa, NJ) pp. 375–390.

GRÜNERT, S., and JACKSON, R.J. (1994). The immediate downstream codon strongly influences the efficiency of utilization of eukaryotic translation initiation codons. EMBO J. **13,** 3618–3630.

GUINN, B., WANG, X.-H., LARAYA, P., and KEATING, A. (1996). Xenotransplantation of electrotransfected marrow stromal cells express human factor IX protein in SCID mice: A pre-clinical model for gene therapy. Blood **88**(10; Suppl. 1), 3921.

HOEBEN, R.C., VAN DER JAGT, R.C., SCHOUTE, F., VAN TILBURG, N.H., VERBEET, M.P., BRIET, E., VAN ORMONDT, H., and VAN DER EB, A.J. (1990). Expression of functional factor VIII in primary human skin fibroblasts after retrovirus-mediated gene transfer. J. Biol. Chem. **265,** 7318–7323.

HOYER, L.W. (1995). Why do so many haemophilia A patients develop an inhibitor? Br. J. Haematol. **90,** 498–501.

HURWITZ, D.R., KIRCHGESSER, M., MERRILL, W., GALANOPOULOS, T., McGRATH, C.A., EMAMI, S., HANSEN, M., CHERINGTON, V., APPEL, J.M., BIZINKAUSKAS, C.B., BRACKMANN, H.H., LEVINE, P.H., and GREENBERGER, J.S. (1997). Systemic delivery of human growth hormone or human factor IX in dogs by reintroduced genetically modified autologous bone marrow stromal cells. Hum. Gene Ther. **8,** 137–156.

HUSS, R., SMITH, F.O., MYERSON, D.H., and DEEG, H.J. (1995). Homing and immunogenicity of murine stromal cells transfected with xenogeneic MHC class II genes. Cell Transplant. **4,** 483–491.

ISRAEL, D.I., and KAUFMAN, R.J. (1990). Retroviral-mediated transfer and amplification of a functional human factor VIII gene. Blood **75,** 1074–1080.

KAUFMAN, R.J. (1992). Biological regulation of factor VIII activity. Annu. Rev. Med. **43,** 325–339.

KAUFMAN, R.J., WASLEY, L.C., and DORNER, A.J. (1988). Synthesis, processing, and secretion of recombinant human factor VIII expressed in mammalian cells. J. Biol. Chem. **263,** 6352–6362.

KOZAK, M. (1989). The scanning model for translation: An update. J. Cell Biol. **108,** 229–241.

KRALL, W.J., SKELTON, D.C., YU, X.J., RIVIERE, I., LEHN, P., MULLIGAN, R.C., and KOHN, D.B. (1996). Increased levels of spliced RNA account for augmented expression from the MFG retroviral vector in hematopoietic cells. Gene Ther. **3,** 37–48.

LABAT-MOLEUR, F., STEFFAN, A.M., BRISSON, C., PERRON, H., FEUGEAS, O., FURSTENBERGER, P., OBERLING, F., BRAMBILLA, E., and BEHR, J.P. (1996). An electron microscopy study into the mechanism of gene transfer with lipopolyamines. Gene Ther. **3,** 1010–1017.

LIND, P., LARSSON, K., SPIRA, J., SYDOW-BACKMAN, M., ALMSTEDT, A., GRAY, E., and SANDBERG, H. (1995). Novel forms

of B-domain-deleted recombinant factor VIII molecules. Construction and biochemical characterization. Eur. J. Biochem. **232,** 19–27.

LYNCH, C.M., ISRAEL, D.I., KAUFMAN, R.J., and MILLER, A.D. (1993). Sequences in the coding region of clotting factor VIII act as dominant inhibitors of RNA accumulation and protein production. Hum. Gene Ther. **4,** 259–272.

MARKOWITZ, D., GOFF, S., and BANK, A. (1988a). A safe packaging line for gene transfer: Separating viral genes on two different plasmids. J. Virol. **62,** 1120–1124.

MARKOWITZ, D., GOFF, S., and BANK, A. (1988b). Construction and use of a safe and efficient amphotropic packaging cell line. Virology **167,** 400–406.

MEULIEN, P., FAURE, T., MISCHLER, F., HARRER, H., ULRICH, P., BOUDERBALA, B., DOTT, K., SAINTE MARIE, M., MAZURIER, C., WIESEL, M.L., VAN DE POL, H., CAZENAVE, J.-P., and PAVIRINI, A. (1988). A new recombinant procoagulant protein derived from the cDNA encoding human factor VIII. Protein Eng. **2,** 301–306.

NOLTA, J.A., HANLEY, M.B., and KOHN, D.B. (1994). Sustained human hematopoiesis in immunodeficient mice by cotransplantation of marrow stroma expressing human interleukin-3: Analysis of gene transduction of long-lived progenitors. Blood **83,** 3041–3051.

PAVIRANI, A., MEULIN, P., HARRER, H., SCHAMBER, F., DOTT, K., VILLEVAL, D., CORDIER, Y., WIESEL, M., MAZURIER, C., VAN DE POL, H., PIQUET, Y., CAZENAVE, J., and LECOCQ, P. (1987). Choosing a host cell for active recombinant factor VIII production using vaccinia virus. Bio/Technology **5,** 987–990.

PEREIRA, R.F., HALFORD, K.W., O'HARA, M.D., LEEPER, D.B., SOKOLOV, B.P., POLLARD, M.D., BAGASRA, O., and PROCKOP, D.J. (1995). Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc. Natl. Acad. Sci. U.S.A. **92,** 4857–4861.

PITTMAN, D.D., and KAUFMAN, R.J. (1988). Proteolytic requirements for thrombin activation of anti-hemophilic factor (factor VIII). Proc. Natl. Acad. Sci. U.S.A. **85,** 2429–2433.

PITTMAN, D.D., ALDERMAN, E.M., TOMKINSON, K.N., WANG, J.H., GILES, A.R., and KAUFMAN, R.J. (1993). Biochemical, immunological, and *in vivo* functional characterization of B-domain-deleted factor VIII. Blood **81,** 2925–2935.

PROCKOP, D.J. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. Science **276,** 71–74.

ROSENDAAL, F.R., SMIT, C., and BRIET, E. (1991). Hemophilia treatment in historical perspective: A review of medical and social developments. Ann. Hematol. **62,** 5–15.

SARVER, N., RICCA, G.A., LINK, J., NATHAN, M.H., NEWMAN, J., and DROHAN, W.N. (1987). Stable expression of recombinant factor VIII molecules using a bovine papillomavirus vector. DNA **6,** 553–564.

THOMPSON, A.R. (1995). Progress towards gene therapy for the hemophilias. Thromb. Haemost. **74,** 45–51.

TOOLE, J.J., KNOPF, J.L., WOZNEY, J.M., SULTZMAN, L.A., BUECKER, J.L., PITTMAN, D.D., KAUFMAN, R.J., BROWN, E., SHOEMAKER, C., ORR, E.C., AMPHLETT, G.W., FOSTER, W.B., COE, M.L., KNUTSON, G.J., FASS, D.N., and HEWICK, R.M. (1984). Molecular cloning of a cDNA encoding human antihaemophilic factor. Nature (London) **312,** 342–347.

TOOLE, J.J., PITTMAN, D.D., ORR, E.C., MURTHA, P., WASLEY, L.C., and KAUFMAN, R.J. (1986). A large region (approximately equal to 95 kDa) of human factor VIII is dispensable for *in vitro* procoagulant activity. Proc. Natl. Acad. Sci. U.S.A. **83,** 5939–5942.

VEHAR, G.A., KEYT, B., EATON, D., RODRIGUEZ, H., O'BRIEN, D.P., ROTBLAT, F., OPPERMANN, H., KECK, R., WOOD, W.I., HARKINS, R.N., TUDDENHAM, E.G.D., LAWN, R.M., and CAPON, D.J. (1984). Structure of human factor VIII. Nature (London) **312,** 337–342.

VLOT, A.J., KOPPELMAN, S.J., BOUMA, B.N., and SIXMA, J.J. (1998). Factor VIII and von Willebrand factor. Thromb. Haemost. **79,** 456–465.

WALTER, J., and HIGH, K.A. (1997). Gene therapy for the hemophilias. Adv. Vet. Med. **40,** 119–134.

Address reprint requests to:
Dr. Gisela G. Chiang
ALG Company
734 Forest Street
Marlborough, MA 01752

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